

**Disinfektan kimia dan antiseptik - Uji kuantitatif
suspensi untuk evaluasi aktivitas *bactericidal* dasar
pada disinfektan kimia dan antiseptik - Metode uji
dan persyaratan
(fase 1)**

**Chemical disinfectants and antiseptics - Quantitative
suspension test for the evaluation of basic bactericidal
activity of chemical disinfectants and antiseptics - Test
method and requirements (phase 1)**

(EN 1040:2005, IDT, Eng)

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Prakata

Standar Nasional Indonesia (SNI) EN 1040:2005, dengan judul *Disinfektan kimia dan antiseptik - Uji kuantitatif suspensi untuk evaluasi aktivitas baktericidal dasar pada disinfektan kimia dan antiseptik - Metode uji dan persyaratan (fase 1)* (EN 1040:2005, IDT, Eng), merupakan hasil adopsi identik dengan metode republikasi *reprint* dari standar EN 1040:2005 *Chemical disinfectants and antiseptics - Quantitative suspension test for the evaluation of basic bactericidal activity of chemical disinfectants and antiseptics - Test method and requirements (phase 1)*, yang ditetapkan oleh BSN pada tahun 2020. Standar ini dirumuskan dalam rangka mendukung penanganan pandemi COVID 19 di Indonesia.

Standar ini disusun oleh Komite Teknis 11-11 Produk Higiene Perbekalan Kesehatan Rumah Tangga dengan Badan Standardisasi Nasional (BSN) sebagai sekretariat Komite Teknis. Standar ini telah dibahas dalam rapat-rapat teknis, dan terakhir disepakati dalam rapat konsensus di Jakarta pada tanggal 28 Mei 2020 yang dihadiri oleh para pemangku kepentingan (*stakeholder*) terkait, yaitu perwakilan dari produsen, konsumen, pakar dan pemerintah, serta perwakilan dari lembaga penguji, asosiasi, perguruan tinggi, pakar serta instansi terkait.

Standar ini telah melalui tahap jajak pendapat pada tanggal 30 Juni sampai dengan 27 Juli 2020 dengan hasil akhir disetujui menjadi SNI.

Untuk tujuan penggunaan standar ini, istilah "*This European Standard*" diganti menjadi "*This standard*".

Apabila di kemudian hari pengguna menemukan kesulitan dalam penggunaan standar ini, maka dianjurkan untuk merujuk pada standar aslinya yaitu EN 1040:2005 dan/atau dokumen terkait lain yang menyertainya.

Perlu diperhatikan bahwa kemungkinan beberapa unsur dari dokumen standar ini dapat berupa hak paten. Badan Standardisasi Nasional tidak bertanggungjawab untuk pengidentifikasian salah satu atau seluruh hak paten yang ada.

Introduction

This Standard specifies a suspension test for establishing whether a chemical disinfectant or antiseptic does or does not have a *basic* bactericidal activity in the fields described in the scope. The acceptability of a product for a defined purpose cannot be determined from this test method. Therefore products are subjected to further testing by relevant tests specified in Standards in order to evaluate their activity under conditions appropriate to their intended use. These Standards have been or will be developed by CEN/TC 216

Disinfektan kimia dan antiseptik - Uji kuantitatif suspensi untuk evaluasi aktivitas *bactericidal* dasar pada disinfektan kimia dan antiseptik - Metode uji dan persyaratan (fase 1)

1 Scope

This standard specifies a test method and the minimum requirements for basic bactericidal activity of chemical disinfectant and antiseptic products that form a homogeneous, physically stable preparation when diluted with water. Products can only be tested at a concentration of 80 % or less as some dilution is always produced by adding the test organisms and water.

This standard applies to active substances (antibacterial biocides) and to formulations under development that are planned to be used in food, industrial, domestic and institutional, medical and veterinary areas. It applies also to the evaluation of bactericidal activity of chemical antiseptics and disinfectants when appropriate standards are not available.

NOTE 1 Standard does not evaluate the activity of a product for an intended use.

NOTE 2 This method corresponds to a phase 1 test (Annex F).

2 Normative reference

The following referenced documents are indispensable for the application of this standard. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

EN 12353, *Chemical disinfectants and antiseptics — Preservation of microbial strains used for the determination of bactericidal and fungicidal activity*

3 Term and definition

For the purposes of this standard, the following terms and definitions apply.

3.1

product

chemical agent or formulation used as chemical disinfectant or antiseptic

3.2

bactericide

product that kills vegetative bacteria under defined conditions

NOTE The adjective derived from "bactericide" is "bactericidal".

3.3

bactericidal activity

capability of a product to produce a reduction in the number of viable bacterial cells of relevant test organisms under defined conditions

3.4

bacteriostatic activity

capability of a product to inhibit the growth of bacteria under defined conditions

4 Requirements

The product shall demonstrate at least a 5 decimal log (lg) reduction when tested in accordance with Clause 5.

The bactericidal activity shall be evaluated using at least the following obligatory experimental test conditions: two test organisms (*Pseudomonas aeruginosa* and *Staphylococcus aureus*), 20 °C, 5 min.

Where indicated, bactericidal activity could be determined applying additional contact times, temperatures and test organisms in accordance with 5.2.1 and 5.5.1.1.

NOTE 1 For these additional conditions, the concentration defined as a result can be lower than the one obtained under the obligatory test conditions.

NOTE 2 At the concentration defined as a result, it is not necessary to demonstrate a 5 lg reduction with the obligatory test conditions.

5 Test methods

5.1 Principle

5.1.1 A sample of the product as delivered (highest test concentration = 80 %) and/or diluted with water is added to a test suspension of bacteria. The mixture is maintained at (20 ± 1) °C for 5 min \pm 10 s (obligatory test conditions). At the end of this contact time, an aliquot is taken; the bactericidal and/or the bacteriostatic activity in this portion is immediately neutralized or suppressed by a validated method. The method of choice is dilution-neutralization. If a suitable neutralizer cannot be found, membrane filtration is used. The numbers of surviving bacteria in each sample are determined and the reduction is calculated.

5.1.2 The test is performed using *Pseudomonas aeruginosa* and *Staphylococcus aureus* as test organisms (obligatory test conditions).

5.1.3 Additional and optional contact times and temperatures are specified. Additional test organisms can be used.

5.2 Materials and reagents

5.2.1 Test organisms

The bactericidal activity shall be evaluated using the following strains as test organisms ¹⁾

— *Pseudomonas aeruginosa* ATCC 15442;

Staphylococcus aureus ATCC 6538.

¹⁾ The ATCC numbers are the collection numbers of strains supplied by the American Type Culture Collection (ATCC). This information is given for the convenience of users of this Standard and does not constitute an endorsement by CEN of the product named

NOTE See Annex A for strain references in some other culture collections.

The required incubation temperature for these test organisms is $(36 \pm 1) ^\circ\text{C}$ or $(37 \pm 1) ^\circ\text{C}$ (5.3.2.3). The same temperature (either $36 ^\circ\text{C}$ or $37 ^\circ\text{C}$) shall be used for all incubations performed during a test and its control and validation.

If additional test organisms are used, they shall be incubated under optimum growth conditions (temperature, time, atmosphere, media) noted in the test report. If the additional test organisms selected do not correspond to the specified strains, their suitability for supplying the required inocula shall be verified. If these additional test organisms are not classified at a reference centre, their identification characteristics shall be stated. In addition, they shall be held by the testing laboratory or national culture collection under a reference for five years.

5.2.2 Culture media and reagents

5.2.2.1 General

All weights of chemical substances given in this standard refer to the anhydrous salts. Hydrated forms may be used as an alternative, but the weights required shall be adjusted to allow for consequent molecular weight differences.

The reagents shall be of analytical grade and/or appropriate for microbiological purposes. They shall be free from substances that are toxic or inhibitory to the test organisms.

NOTE 1 To improve reproducibility, it is recommended that commercially available dehydrated material is used for the preparation of culture media. The manufacturer's instructions relating to the preparation of these products should be rigorously followed.

NOTE 2 For each culture medium and reagent, a limitation for use should be fixed.

5.2.2.2 Water

The water shall be freshly glass-distilled water and not demineralised water.

Sterilise in the autoclave [5.3.2.1 a)].

NOTE 1 Sterilisation is not necessary if the water is used e.g. for preparation of culture media and subsequently sterilised.

NOTE 2 If distilled water of adequate quality is not available, water for injections (see bibliographic reference [1]) can be used.

5.2.2.3 Tryptone Soya Agar (TSA)

Tryptone soya agar, consisting of:

Tryptone, pancreatic digest of casein	15,0 g
Soya peptone, papaic digest of soybean meal	5,0 g
Sodium chloride (NaCl)	5,0 g
Agar	15,0 g
Water (5.2.2.2)	to 1.000,0 ml

Sterilise in the autoclave [5.3.2.1 a)]. After sterilisation, the pH of the medium shall be equivalent to $(7,2 \pm 0,2)$ when measured at $(20 \pm 1) ^\circ\text{C}$.

NOTE In case of encountering problems with neutralization (**5.5.1.2** and **5.5.1.3**) it may be necessary to add neutralizer to the TSA. **Annex B** gives guidance on the neutralizers that may be used.

5.2.2.4 Diluent

Tryptone sodium chloride solution, consisting of:

Tryptone, pancreatic digest of casein	1,0 g
Sodium chloride (NaCl)	8,5 g
Water (5.2.2.2)	to 1.000,0 ml

Sterilise in the autoclave [5.3.2.1a)]. After sterilisation, the pH of the diluent shall be equivalent to $(7,0 \pm 0,2)$ when measured at $(20 \pm 1) ^\circ\text{C}$.

5.2.2.5 Neutralizer

The neutralizer shall be validated for the product being tested in accordance with **5.5.1.2**, **5.5.1.3** and **5.5.2**. It shall be sterile.

NOTE Information on neutralizers that have been found to be suitable for some categories of products is given in Annex B.

5.2.2.6 Rinsing liquid (for membrane filtration)

The rinsing liquid shall be validated for the product being tested in accordance with **5.5.1.2**, **5.5.1.3** and **5.5.3**. It shall be sterile, compatible with the filter membrane and capable of filtration through the filter membrane under the test conditions described in **5.5.3**.

NOTE Information on rinsing liquids that have been found to be suitable for some categories of products is given in Annex B.

5.3 Apparatus and glassware

5.3.1 General

Sterilise all glassware and parts of the apparatus that will come into contact with the culture media and reagents or the sample, except those that are supplied sterile by one of the following methods:

- a) by moist heat, in the autoclave [5.3.2.1 a)];
- b) by dry heat, in the hot air oven [5.3.2.1 b)].

5.3.2 Usual microbiological laboratory equipment²⁾ and, in particular, the following:

5.3.2.1 Apparatus for sterilisation

- a) for moist heat sterilisation, an autoclave capable of being maintained at $(121^{+3}_0) ^\circ\text{C}$ for a minimum holding time of 15 min;
- b) for dry heat sterilisation, a hot air oven capable of being maintained at $(180^{+5}_0) ^\circ\text{C}$ for a minimum holding time of 30 min, at $(170^{+5}_0) ^\circ\text{C}$ for a minimum holding time of 1 h or at $(160^{+5}_0) ^\circ\text{C}$ for a minimum holding time of 2 h.

²⁾ Disposable sterile equipment is an acceptable alternative to reusable glassware.

5.3.2.2 Water baths, capable of being controlled at $(20 \pm 1) ^\circ\text{C}$, at $(45 \pm 1) ^\circ\text{C}$ (to maintain melted TSA in case of pour plate technique) and at additional test temperatures $\pm 1 ^\circ\text{C}$ (5.5.1)

5.3.2.3 Incubator, capable of being controlled at $(36 \pm 1) ^\circ\text{C}$ or $(37 \pm 1) ^\circ\text{C}$ (5.2.1).

5.3.2.4 pH-meter, having an inaccuracy of calibration of no more than $\pm 0,1$ pH units at $(20 \pm 1) ^\circ\text{C}$.

NOTE A puncture electrode or a flat membrane electrode should be used for measuring the pH of the agar media (5.2.2.3)

5.3.2.5 Stopwatch

5.3.2.6 Shakers

a) electromechanical agitator, e.g. Vortex® mixer³⁾;

b) mechanical shaker.

5.3.2.7 Membrane filtration apparatus, constructed of a material compatible with the substances to be filtered.

The apparatus shall have a filter holder of at least 50 ml volume. It shall be suitable for use with filters of diameter 47 mm to 50 mm and 0,45 μm pore for the membrane filtration method (5.5.3).

The vacuum source used shall give an even filtration flow rate. In order to obtain a uniform distribution of the micro-organisms over the membrane and to prevent overlong filtration, the device shall be set so as to obtain the filtration of 100 ml of rinsing liquid in 20 s to 40 s.

5.3.2.8 Refrigerator, capable of being controlled at $2 ^\circ\text{C}$ to $8 ^\circ\text{C}$

5.3.2.9 Graduated pipettes, of nominal capacities 10 ml, 1 ml and 0,1 ml, or calibrated automatic pipettes

5.3.2.10 Petri dishes (plates), of size 90 mm to 100 mm

5.3.2.11 Glass beads, 3 mm to 4 mm in diameter

5.3.2.12 Volumetric flasks

5.4 Preparation of test organism suspensions and product test solutions

5.4.1 Test organism suspensions (test and validation suspension)

5.4.1.1 General

For each test organism, two different suspensions have to be prepared: the “test suspension” to perform the test and the “validation suspension” to perform the controls and method validation.

³⁾ Vortex® is an example of a suitable product available commercially. This information is given for the convenience of users of this standard and does not constitute an endorsement by CEN of this product.

5.4.1.2 Preservation and stock cultures of test organisms

The test organisms and their stock cultures shall be prepared and kept in accordance with EN 12353.

5.4.1.3 Working culture of test organisms

In order to prepare the working culture of the test organism (5.2.1), prepare a subculture from the stock culture (5.4.1.2) by streaking onto TSA (5.2.2.3) slopes or plates and incubate (5.3.2.3). After 18 h to 24 h, prepare a second subculture from the first subculture in the same way and incubate for 18 h to 24 h. From this second subculture, a third subculture may be produced in the same way. The second and (if produced) third subcultures are the working cultures

If it is not possible to prepare the second subculture on a particular day, a 48 h subculture may be used for subsequent subculturing, provided that the subculture has been kept in the incubator (5.3.2.3) during the 48 h period.

Never produce and use a fourth subculture.

For additional test organisms, any departure from this method of culturing the test organisms or of preparing the suspensions shall be noted, giving the reasons in the test report.

5.4.1.4 Test suspension ("N")

- a) Take 10 ml of diluent (5.2.2.4) and place in a 100 ml flask with 5 g of glass beads (5.3.2.11). Take the working culture (5.4.1.3) and transfer loopfuls of the cells into the diluent (5.2.2.4). The cells should be suspended in the diluent by rubbing the loop against the wet wall of the flask to dislodge the cells before immersing in the diluent. Shake the flask for 3 min using a mechanical shaker [(5.3.2.6b)]. Aspirate the suspension from the glass beads and transfer to another tube.
- b) Adjust the number of cells in the suspension to $1,5 \times 10^8$ cfu/ml⁴⁾ to $5,0 \times 10^8$ cfu/ml using diluent (5.2.2.4), estimating the number of cfu by any suitable means. Maintain this test suspension in the water bath at the test temperature θ [5.5.1.1a)] and use within 2 h.

NOTE The use of a spectrophotometer for adjusting the number of cells is highly recommended (about 620 nm wavelength - cuvette 10 mm path length). Each laboratory should therefore produce calibration data for each test organism knowing that suitable values of optical density are generally found between 0,150 and 0,460. A colorimeter is a suitable alternative.

- c) For counting, prepare 10^{-6} and 10^{-7} dilutions of the test suspension using diluent (5.2.2.4). Mix [(5.3.2.6a)]. Take a sample of 1,0 ml of each dilution in duplicate and inoculate using the pour plate or the spread plate technique.
 - 1) When using the pour plate technique, transfer each 1,0 ml sample into separate Petri dishes and add 15 ml to 20 ml melted TSA (5.2.2.3), cooled to $(45 \pm 1) ^\circ\text{C}$.
 - 2) When using the spread plate technique, spread each 1,0 ml sample – divided into portions of approximately equal size – on an appropriate number (at least two) of surface dried plates containing TSA (5.2.2.3).

For incubation and counting, see 5.4.1.6.

⁴⁾ cfu/ml = colony forming unit(s) per millilitre

5.4.1.5 Validation suspension (“N_v”)

- a) To prepare the validation suspension (N_v), dilute the test suspension (5.4.1.4) with the diluent (5.2.2.4) to obtain $3,0 \times 10^2$ cfu/ml to $1,6 \times 10^3$ cfu/ml [about one fourth (1+3) of the 10^{-5} dilution].
- b) For counting prepare a 10^{-1} dilution with diluent (5.2.2.4). Mix [5.3.2.6a)]. Take a sample of 1,0 ml in duplicate and inoculate using the pour plate or the spread plate technique [5.4.1.4 c)].

For incubation and counting see 5.4.1.6.

5.4.1.6 Incubation and counting of the test and the validation suspensions

- a) Incubate (5.3.2.3) the plates for 20 h to 24 h. Discard any plates that are not countable for any reason. Count the plates and determine the number of cfu. Incubate the plates for a further 20 h to 24 h. Do not recount plates that no longer show well-separated colonies. Recount the remaining plates. If the number has increased, use only the higher number for further evaluation.
- b) Note for each plate the exact number of colonies but record > 330 for any counts higher than 330 and determine the V_c values according to 5.6.2.2.
- c) Calculate the numbers of cfu/ml in the test suspension “N” and in the validation suspensions “N_v” using the methods given in 5.6.2.3 and 5.6.2.5. Verify according to 5.7.

5.4.2 Product test solutions

The concentration of a product test solution shall be 1,25 times the desired test concentration because it is diluted to 80 % during the test and the method validation (5.5.2 or 5.5.3). Product test solutions shall be prepared in water (5.2.2.2) at minimum three different concentrations to include one concentration in the active range and one concentration in the non-active range (5.8.2). The product as received may be used as one of the product test solutions, in this case the highest tested concentration is 80 %.

For solid products, dissolve the product as received by weighing at least $1,0 \text{ g} \pm 10 \text{ mg}$ of the product in a volumetric flask and filling up with water (5.2.2.2). Subsequent dilutions (lower concentrations) shall be prepared in volumetric flasks (5.3.2.12) on a volume/volume basis in water (5.2.2.2).

For liquid products, dilutions of the product shall be prepared with water (5.2.2.2) on a volume/volume basis using volumetric flasks (5.3.2.12).

The product test solutions shall be prepared freshly and used in the test within 2 h. They shall give a physically homogeneous preparation that is stable during the whole procedure. If during the procedure a visible inhomogeneity appears due to the formation of a precipitate or flocculant, it shall be recorded in the test report.

NOTE Counting micro-organisms embedded in a precipitate or flocculant is difficult and unreliable.

The concentration of the product stated in the test report shall be the desired test concentration. Record the test concentration in terms of mass per volume or volume per volume and details of the product sample as received.

5.5 Procedure for assessing the bactericidal activity of the product

5.5.1 General

5.5.1.1 Experimental conditions (obligatory and additional)

Besides the obligatory temperature, contact time and test organisms additional experimental conditions may be selected (Clause 4), as follows:

a) temperature θ (in °C):

- the obligatory temperature to be tested is $\theta = 20$ °C;
- the additional temperatures may be chosen from 4 °C, 10 °C or 40 °C;
- the allowed deviation for each chosen temperature is ± 1 °C.

b) contact time t (in min):

- the obligatory contact time to be tested is $t = 5$ min;
- the additional contact times may be chosen from 1 min, 15 min, 30 min or 60 min;
- the allowed deviation for each chosen contact time is ± 10 s, except for 1 min, for which it is ± 5 s.

c) test organism:

- the obligatory test organisms are: *Pseudomonas aeruginosa* and *Staphylococcus aureus*;
- additional test organisms may be tested.

5.5.1.2 Choice of test method (dilution-neutralization or membrane filtration)

The method of choice is the dilution-neutralization method (5.5.2). To determine a suitable neutralizer, carry out the validation of the dilution neutralization method (5.5.2.3, 5.5.2.4 and 5.5.2.5 in connection with 5.5.2.6) using a neutralizer, chosen according to laboratory experience and published data.

If this neutralizer is not valid, repeat the validation test using an alternative neutralizer containing a combination of polysorbate 80 (30 g/l), saponin (30 g/l), L-histidine (1 g/l), lecithin (3 g/l), sodium thiosulphate (5 g/l) in either diluent (5.2.2.4) or phosphate buffer 0,0025 mol/l (Annex B).

If both neutralizers are found to be invalid, the membrane filtration method (5.5.3) may be used in place of the dilution-neutralization method.

NOTE In special circumstances, it may be necessary to add neutralizer to TSA (5.2.2.3)

5.5.1.3 General instructions for validation and control procedures

The neutralization and/or removal of the bactericidal and/or bacteriostatic activity of the product shall be controlled and validated – only for the highest product test concentration – for each of the used test organisms and for each experimental condition (temperature, contact time). These procedures (experimental condition control, neutralizer or filtration control and method

validation) shall be performed at the same time with the test and with the same neutralizer – or rinsing liquid – used in the test.

If because of problems with neutralization a neutralizer has been added to TSA (5.5.1.2) used for the validation and control procedures the TSA used for the test shall contain the same amount of this neutralizer as well

5.5.1.4 Equilibration of temperature

Prior to testing, equilibrate all reagents (product test solutions (5.4.2), test suspension (5.4.1.4), validation suspension (5.4.1.5), diluent (5.2.2.4), water (5.2.2.2) to the test temperature θ [5.5.1.1a]) using the water bath (5.3.2.2). Check that the temperature of the reagents is stabilized at θ .

The neutralizer (5.2.2.5) or the rinsing liquid (5.2.2.6) and water (5.2.2.2) shall be equilibrated at a temperature of $(20 \pm 1) ^\circ\text{C}$

5.5.1.5 Precautions for manipulation of test organisms

Do not touch the upper part of the test tube sides when adding the test- or the validation suspensions (5.4.1).

5.5.2 Dilution-neutralization method ⁵⁾

5.5.2.1 General

The test and the control and validation procedures (5.5.2.2 through 5.5.2.5) shall be carried out at the same time

5.5.2.2 Test "Na" – determination of bactericidal concentrations

The procedure for determining bactericidal concentrations is as follows.

- a) Pipette 1,0 ml of water (5.2.2.2) into a tube. Add 1,0 ml of the test suspension (5.4.1.4). Start the stopwatch immediately, mix [5.3.2.6a)] and place the tube in a water bath controlled at the chosen temperature θ [5.5.1.1a)] for $2 \text{ min} \pm 10 \text{ s}$.

At the end of this time, add 8,0 ml of one of the product test solutions (5.4.2). Restart the stopwatch at the beginning of the addition. Mix [5.3.2.6a)] and place the tube in a water bath controlled at θ for the chosen contact time t [5.5.1.1b)]. Just before the end of t , mix [5.3.2.6a)] again.

- b) At the end of t , take a 1,0 ml sample of the test mixture "Na" and transfer into a tube containing 8,0 ml neutralizer (5.2.2.5) and 1,0 ml water (5.2.2.2). Mix [5.3.2.6a)] and place in a water bath controlled at $(20 \pm 1) ^\circ\text{C}$. After a neutralization time of $5 \text{ min} \pm 10 \text{ s}$, mix [5.3.2.6a)] immediately take a sample of 1,0 ml of the neutralized test mixture "Na" (containing neutralizer, product test solution, test suspension) in duplicate and inoculate using the pour plate or the spread plate technique.

- 1) When using the pour plate technique, transfer each 1,0 ml sample into separate Petri dishes and add 15 ml to 20 ml melted TSA (5.2.2.3), cooled to $(45 \pm 1) ^\circ\text{C}$.

⁵⁾ For a graphical representation of this method, see Annex C

- 2) When using the spread plate technique, spread each 1,0 ml sample – divided in portions of approximately equal size – on an appropriate number (at least two) of surface dried plates containing TSA (5.2.2.3).

For incubation and counting see 5.5.2.6.

- c) Perform the procedure a) and b) using the other product test solutions at the same time.
- d) Perform the procedure a) to c) applying if appropriate – other additional experimental conditions (5.5.1.1).

5.5.2.3 Experimental conditions control “A” (Validation of the selected experimental conditions and/or verification of the absence of any lethal effect in the test conditions)

To validate the selected experimental conditions and/or verify the absence of any lethal effect in the test conditions, the procedure is as follows.

NOTE When the test is performed at the following conditions: *Staphylococcus aureus* or *Pseudomonas aeruginosa*, 20 °C, at any contact time, this control can be skipped.

- a) Pipette 1,0 ml of water (5.2.2.2) into a tube. Add 1,0 ml of the validation suspension (5.4.1.5). Start the stopwatch immediately, mix [5.3.2.6a)] and place the tube in a water bath controlled at θ for 2 min \pm 10 s. At the end of this time add 8,0 ml of water (5.2.2.2). Restart the stopwatch at the beginning of the addition, mix [5.3.2.6a)] and place the tube in a water bath controlled at θ for t . Just before the end of t , mix [5.3.2.6a)] again.
- b) At the end of t , take a sample of 1,0 ml of this mixture “A” in duplicate and inoculate using the pour plate or spread plate technique [5.5.2.2b)].

For incubation and counting see 5.5.2.6.

5.5.2.4 Neutralizer control “B” (Verification of the absence of toxicity of the neutralizer)

To verify the absence of toxicity of the neutralizer, the procedure is as follows.

- a) Pipette 8,0 ml of the neutralizer – used in the test (5.5.2.2) – and 1,0 ml of water (5.2.2.2) into a tube. Add 1,0 ml of the validation suspension (5.4.1.5). Start the stopwatch at the beginning of the addition, mix [5.3.2.6a)], and place the tube in a water bath controlled at $(20 \pm 1)^\circ\text{C}$ for 5 min \pm 10 s. Just before the end of this time, mix [5.3.2.6a)].
- b) At the end of this time take a sample of 1,0 ml of this mixture “B” in duplicate and inoculate using the pour plate or the spread plate technique [5.5.2.2b)].

For incubation and counting see 5.5.2.6.

5.5.2.5 Method validation “C” (Dilution-neutralization validation)

To validate the dilution neutralization method, the procedure is as follows.

- a) Pipette 1,0 ml of water (5.2.2.2) into a tube. Add 1,0 ml of the diluent (5.2.2.4) and then, starting a stopwatch, 8,0 ml of the product test solution only of the highest concentration used in the test (5.5.2.2). Mix [5.3.2.6a)] and place the tube in a water bath controlled at θ for t . Just before the end of t , mix [5.3.2.6a)] again.

- b) At the end of t transfer 1,0 ml of the mixture into a tube containing 8,0 ml of neutralizer (used in 5.5.2.2). Restart the stopwatch immediately, mix [5.3.2.6a)] and place the tube in a water bath controlled at $(20 \pm 1) ^\circ\text{C}$ for $5 \text{ min} \pm 10 \text{ s}$. Add 1,0 ml of the validation suspension (5.4.1.5). Start a stopwatch at the beginning of the addition and mix [5.3.2.6a)]. Place the tube in a water bath controlled at $(20 \pm 1) ^\circ\text{C}$ for $(30 \pm 1) \text{ min}$. Just before the end of this time, mix [5.3.2.6a)] again. At the end of this time take a sample of 1,0 ml of the mixture "C" in duplicate and inoculate using the pour plate or the spread plate technique [5.5.2.2b)].

For incubation and counting see 5.5.2.6.

5.5.2.6 Incubation and counting of the test mixture and the control and validation mixtures

For incubation and counting of the test mixture and the control and validation mixtures, the procedure is as follows.

- Incubate (5.3.2.3) the plates for 20 h to 24 h. Discard any plates which are not countable (for any reason). Count the plates and determine the number of colony forming units. Incubate the plates for a further 20 h to 24 h. Do not recount plates which no longer show well separated colonies. Recount the remaining plates. If the number has increased, use only the higher number for further evaluation.
- Note for each plate the exact number of colonies but record > 330 for any counts higher than 330 and determine the V_c values according to 5.6.2.2.
- Calculate the numbers of cfu/ml in the test mixture "Na" and in the validation mixtures A, B and C using the method given in 5.6.2.4 and 5.6.2.6. Verify according to 5.7

5.5.3 Membrane filtration method⁶⁾

5.5.3.1 General

The test and the control and validation procedures (5.5.3.2 through 5.5.3.5) shall be carried out in parallel and separately for each experimental condition (5.5.1.1).

Each membrane filtration apparatus shall be equipped with a membrane of $0,45 \mu\text{m}$ pore size and 47 mm to 50 mm diameter (5.3.2.7) and filled with 50 ml of the rinsing liquid (5.2.2.6). The time required for filtering – if longer than one minute in exceptional cases – shall be recorded in the test report. When transferring the membranes to the surface of an agar plate, care should be taken to ensure that the test organisms are on the upper side of the membrane when placed on the plate and to avoid trapping air between the membrane and agar surface.

5.5.3.2 Test "Na" (Determination of the bactericidal concentrations)

The procedure for determining the bactericidal concentrations is as follows.

- See 5.5.2.2a).
- At the end of t take a sample of 0,1 ml of the test mixture "Na" in duplicate and transfer each 0,1 ml sample into a separate membrane filtration apparatus (5.5.3.1). Filter immediately. Filter through at least 150 ml but no more than 500 ml of rinsing liquid (5.2.2.6). If the rinsing liquid is not water, complete the procedure by filtering 50 ml of water (5.2.2.2). Then transfer each of the membranes to the surface of separate TSA plates.

⁶⁾ For a graphical representation of this method see annex C2

For incubation and counting see 5.5.3.6.

- c) See 5.5.2.2c).
- d) See 5.5.2.2d).

5.5.3.3 Experimental conditions control “A” (Validation of the selected experimental conditions and/or verification of the absence of any lethal effect in the test conditions)

To validate the selected experimental conditions and/or verify the absence of any lethal effect in the test conditions, the procedure is as follows.

NOTE When the test is performed at the following conditions: *Staphylococcus aureus* or *Pseudomonas aeruginosa*, 20 °C, at any contact time, this control can be skipped.

- a) See 5.5.2.3a).
- b) At the end of t, take a sample of 1,0 ml of this mixture “A” in duplicate and transfer each 1,0 ml sample into a separate membrane filtration apparatus (5.5.3.1). Filter immediately and additionally 50 ml of water (5.2.2.2). Then transfer each of the membranes to the surface of separate TSA plates (5.2.2.3).

For incubation and counting see 5.5.3.6

5.5.3.4 Filtration control “B” (Validation of the filtration procedure)

To validate the filtration procedure proceed as follows.

Take 0,1 ml of the validation suspension (5.4.1.5) in duplicate (suspension for control “B”) and transfer each 0,1 ml sample into a separate membrane filtration apparatus (5.5.3.1). Filter immediately. Filter through the rinsing liquid (5.2.2.6) the same way as in the test [5.5.3.2b)].

If the rinsing liquid is not water, complete the procedure by filtering 50 ml of water (5.2.2.2). Then transfer each of the membranes to the surface of separate TSA plates (5.2.2.3).

For incubation and counting see 5.5.3.6.

5.5.3.5 Method validation “C” (Validation of the membrane filtration method or counting of the bacteria on the membranes which have previously been in contact with the product)

For validation of the membrane filtration method or counting of the bacteria on the membranes which have previously been in contact with the product, the procedure is as follows.

- a) See 5.5.2.5a).
- b) At the end of t take 0,1 ml of the validation mixture “C” in duplicate and transfer each 0,1 ml sample into a separate membrane filtration apparatus (5.5.3.1). Filter immediately. Filter through the rinsing liquid (5.2.2.6) the same way as in the test [5.5.3.2b)]. Then cover the membranes with 50 ml of the rinsing liquid (5.2.2.6) and add 0,1 ml of the validation suspension (5.4.1.5). Filter immediately again and additionally 50 ml of water (5.2.2.2). Then transfer each of the membranes to the surface of separate TSA plates (5.2.2.3).

For incubation and counting see 5.5.3.6.

5.5.3.6 Incubation and counting of the test mixture and the control and validation mixtures

For incubation and counting of the test mixture and the control and validation mixtures, the procedure is as follows.

- Incubate (5.3.2.3) the plates for 20 h to 24 h. Discard any plates which are not countable (for any reason). Count the plates and determine the number of colony forming units. Incubate the plates for a further 20 h to 24 h. Do not recount plates which no longer show well separated colonies. Recount the remaining plates. If the number has increased use only the higher number for further evaluation.
- Note for each plate the exact number of colonies but record > 165 for any counts higher than 165 and determine the V_C values according to 5.6.2.2.
- Calculate the numbers of cfu/ml in the test mixture N_a and in the validation mixtures A , B and C using the method given in 5.6.2.4 and 5.6.2.6. Verify according to 5.7

5.6 Experimental data and calculation

5.6.1 Explanation of terms and abbreviations

5.6.1.1 Overview of the different suspensions and test mixtures

N and N_v represent the bacterial suspensions, N_a represents the bactericidal test mixture, A (experimental conditions control), B (neutralizer or filtration control), C (method validation) represent the different control test mixtures.

N , N_v , N_0 , N_{v0} , N_a , and A , B and C represent the number of cells counted per ml in the different test mixtures in accordance with Table 1.

Table 1 — Number of cells counted per ml in the different test mixtures

	Number of cells per ml in the bacterial suspensions	Number of cells per ml in the test mixtures at the beginning of the contact time ($time = 0$)	Number of survivors per ml in the test mixtures at the end of the contact time t or 5 min (B) or 30 min (C)
Test	N Test suspension	$N_0 (= N/10)$	N_a (before neutralization or filtration)
Controls	N_v Validation suspension	$N_{v0} (= N_v/10)$	A, B, C

5.6.1.2 V_C -values

All experimental data are reported as V_C values:

- in the dilution-neutralization method (test and controls), a V_C value is the number of colony-forming units counted per 1,0 ml sample;
- in the membrane filtration method, a V_C value is the number of colony-forming units counted per 0,1 ml sample of test mixture N_a and per 1,0 ml sample in the controls.

5.6.2 Calculation

5.6.2.1 General

The first step in the calculation is the determination of the V_C values, the second the calculation of N , N_0 , N_a , N_v , N_{vo} , A , B and C . The third step is the calculation of the reduction R (5.8).

5.6.2.2 Determination of V_C -values

The V_C -values are determined as follows.

- a) The usual limits for counting bacteria on agar plates are between 15 and 300. In this Standard, a deviation of 10 % is accepted, so the limits are 14 and 330. On membranes the usual upper limits are different: 150, i.e. with the 10% deviation: 165.

NOTE The lower limit (14) is based on the fact that the variability increases the smaller the number counted in the sample (1 ml or 0,1 ml) is and therefore subsequent calculations may lead to wrong results. The lower limit refers only to the sample (and not necessarily to the counting on one plate), e.g. three plates per 1 ml sample with 3 cfu, 8 cfu and 5 cfu give a V_C -value of 16.

The upper limit (330, 165) reflects the imprecision of counting confluent colonies and growth inhibition due to nutriment depletion. They refer only to the counting on one plate and not necessarily to the sample.

- b) For counting the test suspension N (5.4.1.6), the validation suspension N_v (5.4.1.6) and for all countings of the dilution-neutralization method (5.5.2.6), determine and record the V_C values according to the number of plates used per 1 ml sample (5.6.1.2).

NOTE If more than one plate per 1 ml sample has been used to determine the V_C value, the countings per plate should be noted.

If the count on one plate is higher than 330, report the number as "> 330". If more than one plate per 1 ml sample has been used and at least one of them shows a number higher than 330, report this V_C value as "> sum of the counts" (e.g. for ">330, 310, 302", report "> 942").

If a V_C value is lower than 14, report the number (but substitute by "<14" for further calculation (in the case of N_a)).

For the membrane-filtration method (5.5.3), the countings on the membranes are the V_C values (5.6.1.2). Report the V_C values below the lower limit (14) or above the upper limit (165) as described above.

- c) Only V_C values within the respective counting limits are taken into account for further calculation, except in the case of N_a (5.6.2.4).

5.6.2.3 Calculation of N and N_0

N is the number of cells per ml in the test suspension (5.4.1.4; 5.6.1.1).

Since the two dilutions of the test suspension (5.4.1.4 in connection with 5.4.1.6) are evaluated, calculate the number of cfu/ml as the weighted mean count using the following formula:

$$N = \frac{c}{(n_1 + 0,1 n_2) 10^{-6}} \quad (1)$$

where

- C is the sum of V_C -values taken into account;
 n_1 is the number of V_C -values taken into account in the lower dilution, i.e. 10^{-6} ;
 n_2 is the number of V_C -values taken into account in the higher dilution, i.e. 10^{-7} ;
 10^{-6} is the dilution factor corresponding to the lower dilution, in this example 10^{-6} .

Round off the results calculated to two significant figures. For this, if the last figure is below 5, the preceding figure is not modified; if the last figure is more than 5, the preceding figure is increased by one unit; if the last figure is equal to 5, round off the preceding figure to the next nearest even figure. Proceed stepwise until two significant figures are obtained. As a result, the number of cfu/ml is expressed by a number between 1,0 and 9,9 multiplied by the appropriate power of 10.

EXAMPLE $N = \frac{168+213+20+25}{(2+0,1 \times 2) 10^{-6}} = \frac{426}{2,2 \times 10^{-6}} = 1,9363 \times 10^8 = 1,9 \times 10^8 \text{ (cfu/ml)}$

N_0 is the number of cells per ml in the test mixture [5.5.2.2a)] at the beginning of the contact time (time “zero” = 0). It is one-tenth of the weighted mean of N due to the tenfold dilution by the addition of the product and water.

5.6.2.4 Calculation of N_a

N_a is the number of survivors per ml in the test mixture [5.5.2.2a) or 5.5.3.2a)] at the end of the contact time and before neutralization or membrane filtration. It is tenfold higher than the V_c values due to the addition of neutralizer and water [5.5.2.2b)] or the sample volume of 0,1 ml [5.5.3.2b)] in the membrane filtration method.

Calculate N_a using the following equation:

$$N_a = 10 c/n \quad (2)$$

where

- c is the sum of V_C -values taken into account;
 n is the number of V_C -values taken into account.

If one or both of the duplicate V_c values are either below the lower or above the upper limit, express the results as “less than” or “more than”.

Examples:

- a) duplicate V_C values: 2, 16

$$N_a = \frac{(< 14 + 16) \times 10}{2} = i. e. < 150$$

- b) duplicate V_C values (membrane filtration): >165, >165

$$N_a = \frac{(> 165 + > 165) \times 10}{2} = i. e. < 1.650$$

- c) duplicate V_C values (two spread plates per 1,0 ml sample): > 660, 600

$$Na = \frac{(> 660 + 600) \times 10}{2} = i. e. < 6.300$$

5.6.2.5 Calculation of N_v and N_{v0}

N_v is the number of cells per ml in the validation suspension [5.4.1.5a)]. It is tenfold higher than the counts in terms of V_C values due to the dilution step of 10^{-1} [5.4.1.5b)].

N_{v0} is the number of cells per ml in the mixtures A, B and C at the beginning of the contact time (time 0) (5.6.1.1). It is one-tenth of the mean of the V_C values of N_v [5.4.1.6c)] taken into account.

Calculate N_v and N_{v0} using the following equations:

$$N_v = 10 c/n \quad (3)$$

$$N_{va} = c/n \quad (4)$$

where

c is the sum of V_C -values taken into account;
 n is the number of V_C -values taken into account.

5.6.2.6 Calculation of A , B and C

A , B and C are the numbers of survivors in the experimental conditions control A (5.5.2.3 or 5.5.3.3), neutralizer control B (5.5.2.4) or filtration control (5.5.3.4) and method validation C (5.5.2.5 or 5.5.3.5) at the end of the contact time t (A) or the defined times 5 min (B) and 30 min (C). They correspond to the mean of the V_C values of the mixtures A, B and C taken into account.

Calculate A , B and C using the following equation:

$$A, B, C = c/n$$

where

c is the sum of V_C -values taken into account;
 n is the number of V_C -values taken into account.

5.7 Verification of the methodology

5.7.1 General

A test is valid if:

- all results meet the criteria of 5.7.3; and
- requirements of 5.8.2 are fulfilled.

5.7.2 Control of weighted mean counts

For results calculated by weighted mean of two subsequent dilutions (e.g. "N"), the quotient of the two means shall not be higher than 15 and not lower than 5. Results below the lower limit are taken as the lower limit number (14). Results above the respective upper limit [5.6.2.2b)] are taken as the upper limit number.

EXAMPLE

For N: 10^{-6} dilution: 168 + 215 cfu/ml, 10^{-7} dilution: 20 + < 14 cfu/ml; $(168 + 215) / (20 + 14) = 383/34 = 11,26 =$ between 5 and 15.

NOTE When the counts obtained on plates are out of the limits fixed for the determination of V_c values [5.6.2.2b)], check for the weighted mean as mentioned above but use only the V_c values within the counting limits for calculation of N .

5.7.3 Basic limits

For each test organism check that:

- a) N is between $1,5 \times 10^8$ and $5,0 \times 10^8$ ($8,17 \leq \lg N \leq 8,70$)
- b) N_0 is between $1,5 \times 10^7$ and $5,0 \times 10^7$ ($7,17 \leq \lg N_0 \leq 7,70$)
 N_{V_0} is between 30 and 160 ($3,0 \times 10^1$ and $1,6 \times 10^2$)
 $(N_{V_B}$ is between $3,0 \times 10^2$ and $1,6 \times 10^3$)
- c) A, B, C are equal to or greater than $0,5 \times N_{V_0}$
- d) control of weighted mean counts (5.7.2): quotient is not lower than 5 and not higher than 15.

5.8 Expression of results and precision

5.8.1 Reduction

The reduction ($R = N_0/N_a$) is expressed in logarithm.

For each test organism record the number of cfu/ml in the test suspension N (5.6.2.3) and in the test N_a (5.6.2.4). Calculate N_0 (5.6.2.3).

For each product concentration and each experimental condition, calculate and record the decimal log reduction (\lg) separately using the equation:

$$\lg R = \lg N_0 - \lg N_a$$

For the controls and validation of the dilution-neutralization method or membrane filtration method, record N_{V_0} (5.6.2.5), the results of A, B and C (5.6.2.6) and their comparison with N_{V_0} [5.7.3c)].

5.8.2 Control of active and non-active product test solution (5.4.2)

At least one concentration per test [5.5.2.2a) - c) or 5.5.3.2a) - c)] shall demonstrate a 5 lg or more reduction and at least one concentration shall demonstrate a lg reduction of less than 5.

5.8.3 Limiting test organism and bactericidal concentration

For each test organism, record the lowest concentration of the product which passes the test ($\lg R \geq 5$). Record as the limiting test organism the test organism requiring the highest of these concentrations (it is the least susceptible to the product in the chosen experimental conditions).

The lowest concentration of the product active on the limiting test organism is the bactericidal concentration determined according to this standard.

5.8.4 Precision, replicates

Taking into account the precision of the methodology determined by a statistical analysis based on data provided by a collaborative study, replication of the test (six replicates for a precision of $\pm 1 \lg$ in reduction) is recommended (Annex E). The number of replicate tests shall be decided according to the required level of precision, taking into account the intended use of the test results.

Replication means the complete test procedure with separately prepared test - and validation suspensions. The replicate of the test may be restricted to the limiting test organism. The mean of the results of the replicates - not each single result - shall demonstrate at least a 5 lg reduction and shall also be calculated and recorded.

5.9 Interpretation of results - Conclusion

According to the chosen experimental conditions (obligatory or obligatory and additional) the bactericidal concentrations determined according to this standard may differ (**Clause 4**).

The product shall be deemed to have passed the EN 1040 standard for bactericidal activity if it demonstrates in a valid test at least a 5 lg reduction in one of the test conditions defined by this standard.

The bactericidal concentration determined according to this standard is reported with the test conditions (temperature, contact time, test organisms).

The *basic* bactericidal concentration according to this standard is the concentration active on the limiting test organism when the test is performed with the obligatory conditions (20 °C, 5 min, test organisms: *Pseudomonas aeruginosa* and *Staphylococcus aureus*).

A product which passes the test is characterized as possessing bactericidal activity in the conditions of the test. In order to qualify the product for a defined purpose it shall be evaluated using additional standard tests which are appropriate to its intended use.

Information regarding the additional standard tests which shall be used to qualify a product as antiseptic and/or disinfectant for a defined purpose is given in **Annex F**

5.10 Test report

The test report shall refer to this standard (EN 1499).

The test report shall state, at least, the following information:

- a) identification of the testing laboratory;
- b) identification of the client;

- c) identification of the sample:
 - 1) name of the product;
 - 2) batch number and - if available - expiry date;
 - 3) manufacturer – if not known: supplier;
 - 4) date of delivery;
 - 5) storage conditions;
 - 6) product diluent recommended by the manufacturer for use;
 - 7) active substance(s) and their concentration(s) (optional);
 - 8) appearance of the product;
- d) test method and its validation:
 - 1) If the dilution-neutralization method is used, full details of the test for validation of the neutralizer shall be given;
 - 2) If the membrane filtration method is used, full details of the procedure which was carried out in order to justify the use of the membrane filtration method shall be given;
- e) experimental conditions:
 - 1) date(s) of test (period of analysis);
 - 2) diluent used for product test solution (distilled water);
 - 3) product test concentrations (= *desired* test concentrations according to 5.4.2);
 - 4) appearance product dilutions;
 - 5) contact time(s);
 - 6) test temperature(s);
 - 7) stability and appearance of the mixture during the procedure (note the formation of any precipitate or flocculant);
 - 8) temperature of incubation;
 - 9) neutralizer or rinsing liquid;
 - 10) identification of the bacterial strains used
- f) test results;
 - 1) controls and validation;
 - 2) evaluation of bactericidal activity;

- 3) number of replicates per test organism;
- g) special remarks
- h) conclusion;
- i) locality, date and identified signature.

NOTE An example of a typical test report is given in Annex D.

Annex A
(informative)
Referenced strains in national collections

— <i>Pseudomonas aeruginosa</i> :	ATCC	15442
	CIP	103467
	DSM	939
	NCIMB	1421
— <i>Staphylococcus aureus</i> :	ATCC	6538
	CIP	483
	DSM	799
	NCTC	10788
	NCIMB	9518

Annex B
(informative)
Suitable neutralizers and rinsing liquids

B.1 General

The weights given in **B.2** to **B.4** refer to the anhydrous salts (5.2.2.1).

The lists in **B.2** to **B.4** are not exhaustive and other reagents may be used.

B.2 Neutralizers

Any of the following neutralizers may be used:

- lecithin 3 g/l; polysorbate 80⁷⁾ 30 g/l; sodium thiosulphate ($\text{Na}_2\text{S}_2\text{O}_3$) 5 g/l; L-histidine 1 g/l; saponin 30 g/l in diluent (5.2.2.4) or in phosphate buffer 0,0025 mol/l;
- phosphate buffer 0,25 mol/l:
 - potassium dihydrogen phosphate (KH_2PO_4) 34 g;
 - water (5.2.2.2) 500 ml;
 - adjusted to pH ($7,2 \pm 0,2$) with sodium hydroxide (NaOH) 1 mol/l;
 - water (5.2.2.2) up to 1 000 ml;
 - sterilized in an autoclave (5.3.1);
- fresh egg yolk diluted to 5 % or 0,5 % (v/v);
- 30 g/l polysorbate 80; 4 g/l sodium dodecyl sulphate ($\text{C}_{12}\text{H}_{25}\text{NaO}_4\text{S}$); lecithin 3 g/l;
- fresh egg yolk diluted to 5 % (v/v); 40 g/l polysorbate 80;
- 7 % (v/v) ethylene oxide condensate of fatty alcohol; 20 g/l lecithin; 4 % (w/v) polysorbate 80;
- 4 % (v/v) ethylene oxide condensate of fatty alcohol; 4 g/l lecithin;
- 30 g/l polysorbate 80; lecithin 3 g/l; L-histidine 1 g/l;
- glycine as a function of the product concentration;
- 30 g/l polysorbate 80; lecithin 3 g/l;
- phospholipid emulsion (commercial) at 50 mg/ml (diluted 1 to 10);

⁷⁾ Analytical quality, non-hydrolyzed in accordance with the European Pharmacopoeia. TWEEN 80 is an example of a suitable product available commercially. This information is given for the convenience of users of this European Standard and does not constitute an endorsement by CEN of this product

- sodium thioglycollate at 0,5 g/l or 5 g/l;
- L-cysteine at 0,8 g/l or 1,5 g/l;
- thiomalic acid at 0,075 % (v/v) - adjusted to pH 7,0 with sodium hydroxide (NaOH);
- sodium thiosulphate at 5 g/l;
- catalase or peroxidase: one unit (U) of these enzymes catalyzes at $(25 \pm 1) ^\circ\text{C}$ the decomposition of 1 μmol of hydrogen peroxide per minute at pH 7,0;
- polysorbate 80 30 g/l; saponin 30 g/l; L-histidine 1 g/l; L-cysteine 1 g/l.

B.3 Rinsing liquids

Any of the following rinsing liquids may be used:

- water (5.2.2.2);
- diluent (5.2.2.4);
- aqueous solution of 0,1 % (w/v) polysorbate 80;
- aqueous solution of 0,5 % (w/v) polysorbate 80;
- aqueous solution of 0,5 % (w/v) polysorbate 80 and 0,7 g/l lecithin;
- neutralizer. Check first if the time required for filtration exceeds 1 min;
- buffer solutions.

B.4 Neutralizer added to the agar for counting

Any of the following neutralizers may be used:

- 10 % (v/v) of a solution containing 0,7 g/l lecithin and 5 % (w/v) polysorbate 80;
- 10 % (v/v) of a solution containing 10 g/l lecithin and 5 % (w/v) polysorbate 80;
- 10 % (v/v) of a solution containing fresh egg yolk 1,5 % (v/v) and 5 % (w/v) polysorbate 80

Annex C (informative) Graphical representation of test procedures

C.1 Dilution-neutralization method

Test (Na)

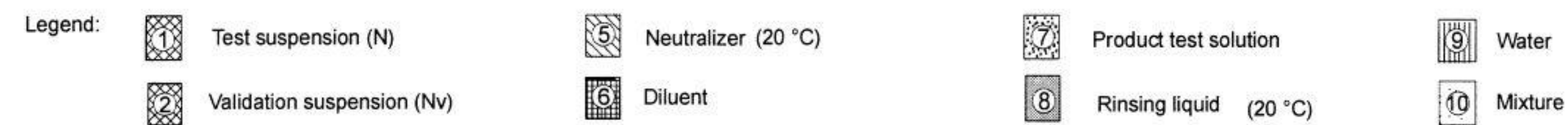
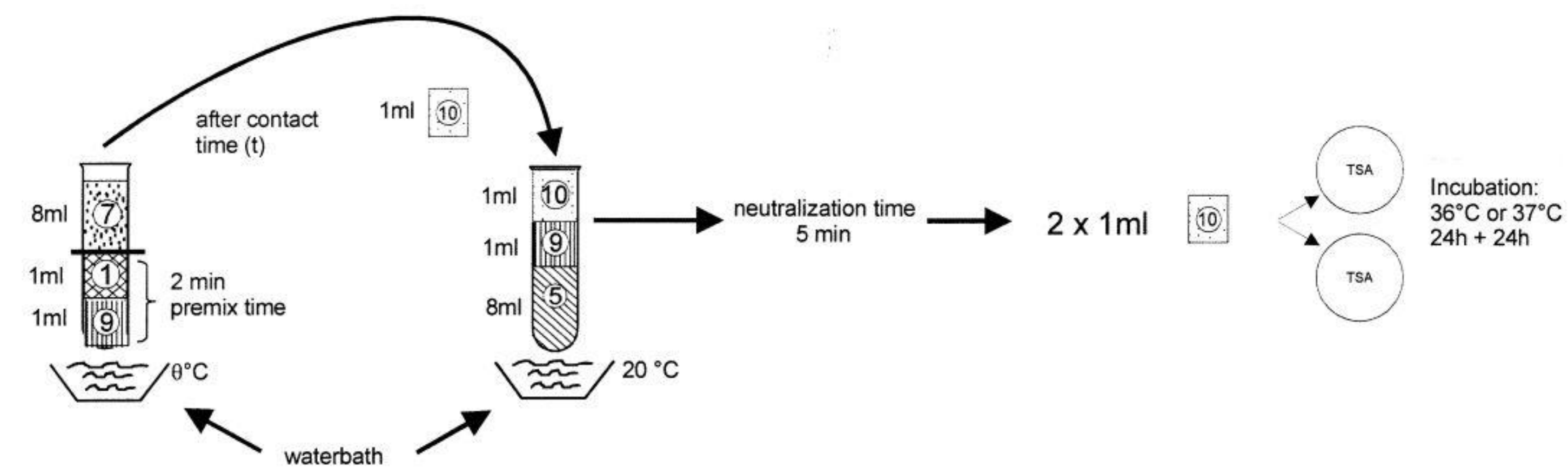


Figure C.1

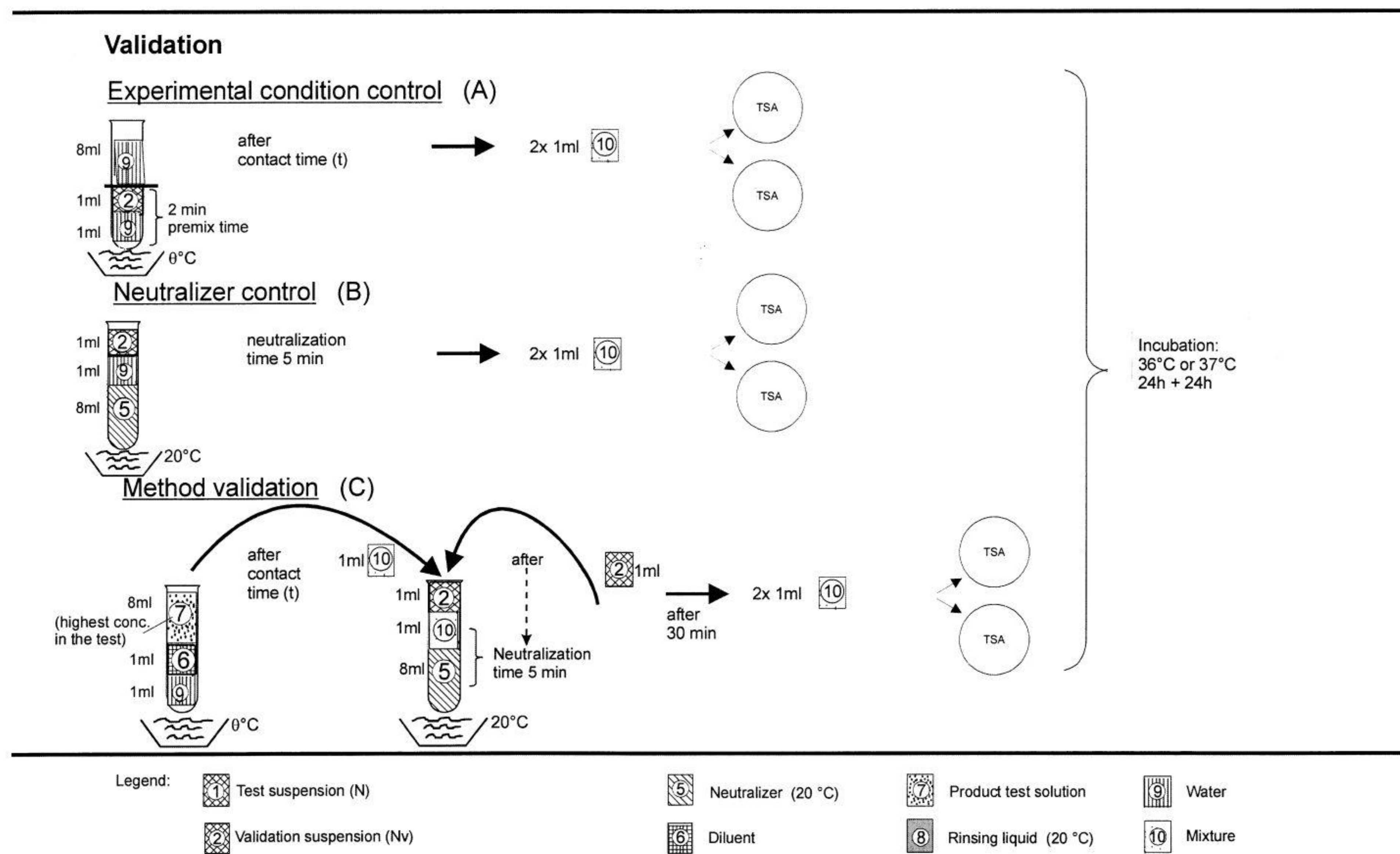
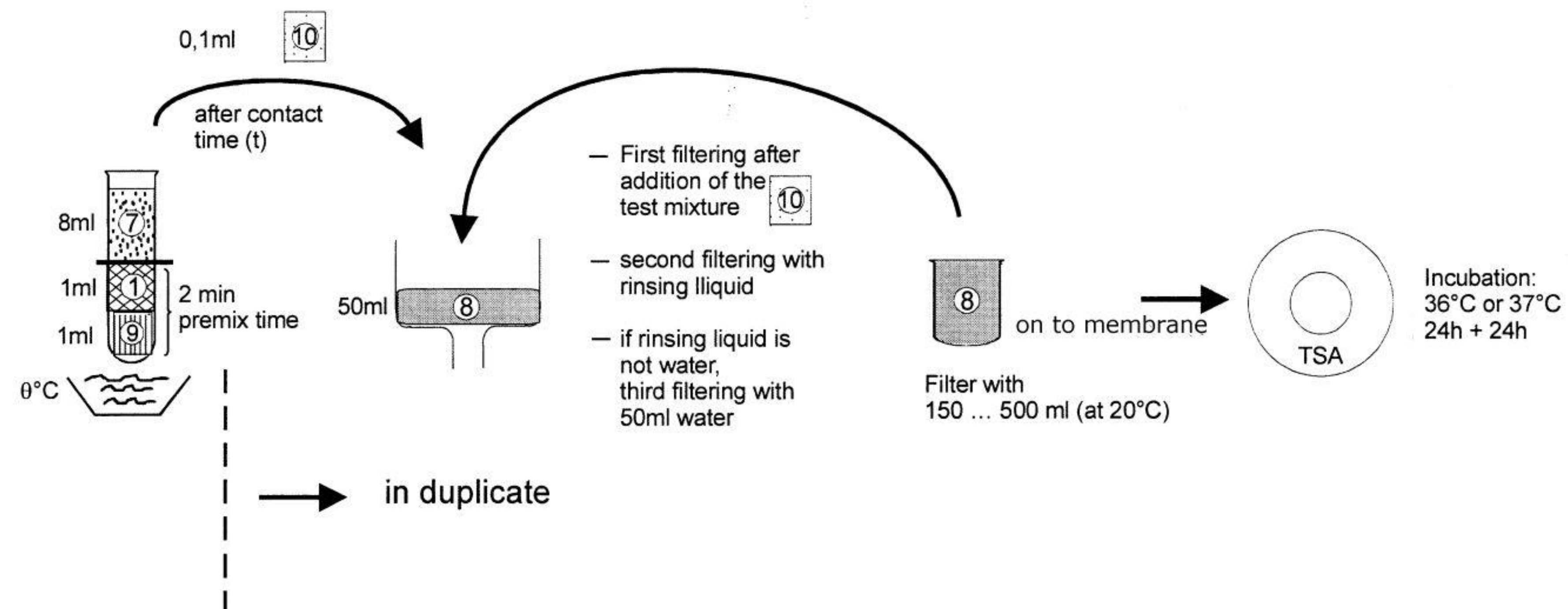


Figure C.2

C.2 Membrane filtration method

Test (Na)



Legend:

Test suspension (N)
 Validation suspension (Nv)

Neutralizer (20 °C)
 Diluent

Product test solution
 Rinsing liquid (20 °C)

Water
 Mixture

Figure C.3

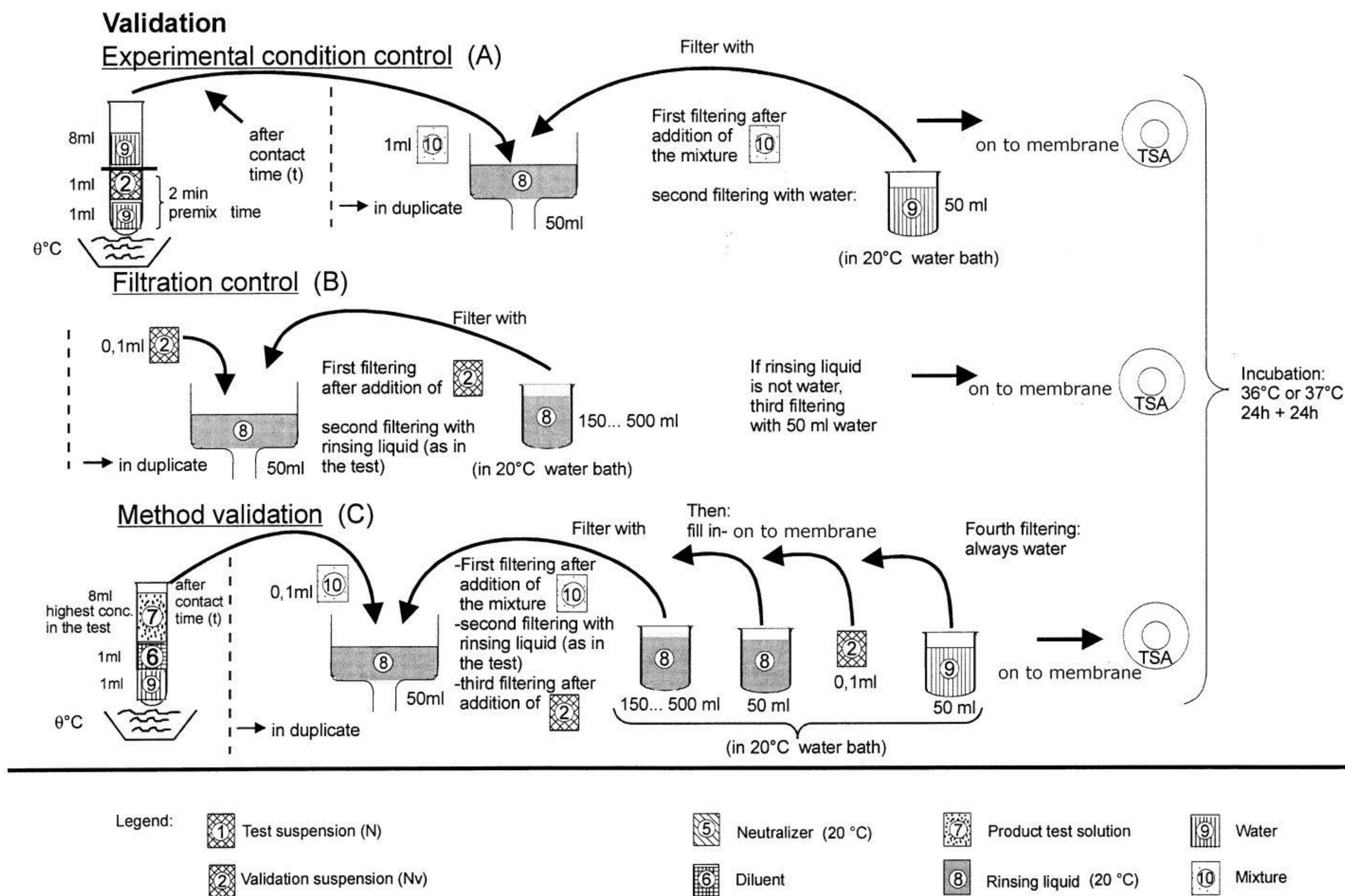


Figure C.4

Annex D
(informative)
Example of a typical test report

NOTE 1 All names and examples in Annex D are fictitious apart from those used in this European Standard.

NOTE 2 Only the test results of one replicate for *Pseudomonas aeruginosa* are given as an example.

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TEST REPORT

EN 1040, BACTERICIDAL ACTIVITY
(obligatory and additional conditions)

Client: Centipede Formulations Inc., Markkleeberg / Euroland

Disinfectant-sample

Name of the product: W **Batch number:** 01-05-48

Manufacturer or - if not known - supplier: Centipede Formulations Inc. (manufacturer) **Storage conditions (temp. and other):** Room temperature, darkness

Appearance of the product: Liquid, clear, yellowish

Active substance(s) and their concentration(s): Not indicated

Product diluent recommended by the manufacturer for use: Potable water

Period of testing

Date of delivery of the product: 2005-05-01 **Dates of tests:** see "Test results" (attached)

Experimental conditions

Product diluent: distilled water ; **concentrations of the product tested:** see "Test results" (attached)

Obligatory conditions: **test-organisms:** *Pseudomonas aeruginosa* ATCC 15442 and *Staphylococcus aureus* ATCC 6538; **test temperature:** 20 °C; **contact time:** 5 min; **Incubation temperature:** 36 °C

Additional conditions:

test organism: *Pseudomonas aeruginosa* ATCC 15442;

Test temperature: 10 °C; **contact time:** 60 min;

Incubation temperature: 36 °C

Test results: see attached sheets.

Special remarks regarding the results:

All controls and validation were within the basic limits.

At least one concentration of the product demonstrated a lg reduction of less than 5 lg.

No precipitate during the test procedure (test mixtures were homogeneous).

Conclusion:

For the product W (batch 01-05-48), the basic bactericidal concentration determined according to the EN 1040 standard (obligatory conditions) is:

1 % (v/v)

(the mean reduction of six replicates with the limiting test organism *Pseudomonas aeruginosa* was $1,2 \times 10^5$. *Staphylococcus aureus* was tested once and showed a 5 lg reduction or more at a lower concentration than *Pseudomonas aeruginosa*).

For the product W (batch 01-05-48), the bactericidal concentration determined according to the EN 1040 standard at 10°C, with 60 min contact time, using *Pseudomonas aeruginosa* ATCC 15442 as test organism is :

1,5 % (v/v).

The product W possesses bactericidal activity in the conditions of the test. In order to qualify the product for a defined purpose it shall be evaluated using additional standard tests which are appropriate to its intended use.

Antiseptville, 2005-10-10

Alexandra May, MD, PhD, Scientific Director

Test results (bactericidal suspension test)

EN...1040.....(Phase 1) Product-name:W..... Batch No.: 01-05-48.....

Remarks:

Dilution neutralization method ☒ Pour plate ☐ Spread plate ☒ Number of plates2 / ml

Neutralizer: ...Leiothin 3,0 g/l in diluent.....

Membrane filtration method Rinsing liquid:.....

Test temperature: 20 °C

Test organism: ...Pseudomonas aeruginosa ATCC 15442.....Incubation temperature: 36°C

Internal lab. No.: QS 68/00..... Date of test: 2005-05-05 Responsible person: Fang..... Signature: Fang

Diluent used for product test solutions:distilled water. Appearance of the product test solutions:clear...

Validation and controls

Validation suspension (Nvo)			Experimental conditions control (A)			Neutralizer or filtration control (B)			Method validation (C) Product conc.: 10 ml/l		
Vc1	86 (40 + 46)	$\bar{x} =$	Vc1	79 (43 + 36)	$\bar{x} =$	Vc1	86 (42 + 44)	$\bar{x} =$	Vc1	75 (35 + 40)	$\bar{x} =$
Vc2	92 (47 + 45)	89	Vc2	84 (39 + 45)	81,5	Vc2	91 (43 + 48)	88,5	Vc2	87 (41 + 46)	81
30 ≤ \bar{x} of Nvo ≤ 180 ?			\bar{x} of A is ≥ 0,5x \bar{x} of Nvo ?			\bar{x} of B is ≥ 0,5x \bar{x} of Nvo ?			\bar{x} of C is ≥ 0,5x \bar{x} of Nvo ?		
<input checked="" type="checkbox"/> yes <input type="checkbox"/> no			<input checked="" type="checkbox"/> yes <input type="checkbox"/> no			<input checked="" type="checkbox"/> yes <input type="checkbox"/> no			<input checked="" type="checkbox"/> yes <input type="checkbox"/> no		

Test suspension and Test

Test-suspension (N and No):	N	Vc1	Vc2	\bar{x} wm = 193,64 × 10 ⁸ , lg N = 8,29
	10 ⁻⁶	168	213	No = N/10; lg No = 7,29
	10 ⁻⁷	20	25	7,17 ≤ lg No ≤ 7,70? <input checked="" type="checkbox"/> yes <input type="checkbox"/> no

Conc. of the product %	Vc1	Vc2	Na = \bar{x} × 10	lg Na	lg R (No=7,29)	Contact-time (min)
0,50	>660	>630	>6450	>3,81	<3,48	5 min
0,75	122	154	1390	3,14	4,15	5 min
1,00	7	0	<140	<2,15	>5,14	5 min

Countings per plate, for:

N 10⁻⁶: 80 + 88; 105 + 10810⁻⁷: 9 + 11; 15 + 10

Na 0,75 % : 66 + 56; 71 + 83

1,00 % Vc1: 1 + 6

Explanation:

Fc = count per ml (one plate or more)

 \bar{x} = average of Vc1 and Vc2 (1. + 2. duplicate) \bar{x} wm = weighted mean of \bar{x} R = reduction (lg R = lg N₀ - lg Na)

Annex E (informative) Precision of the test result

A collaborative study was carried out to determine the precision of the test method between different laboratories. The study was carried out in 24 laboratories in different European countries. In each laboratory the test was carried out up to 10 times on separate days.

The tests were performed using the dilution neutralization and membrane filtration methods using *Pseudomonas aeruginosa* as the test organism. The products used for the study were benzalkonium chloride and phenol.

The agreement between laboratories expressed in terms of the bactericidal effect (reduction in viable count

≥ 5 log) is very good at low and high concentrations but less good at intermediate levels (Tables E.1 and E.2).

Table E.1 - Percentage of laboratories having more than half of their valid replicates with at least a 10^5 reduction at a given Phenol concentration

	Concentration of phenol %		
Test method	0,5	1,0	2,0
Membrane filtration	0	30	100
Dilution neutralisation	0	5	94

Table E.2 - Percentage of laboratories having more than half their valid replicates with at least a 10^5 reduction at a given Benzalkonium Chloride concentration

	Concentration of benzalkonium chloride		
Test method	0,001	0,004	0,008
Membrane filtration	10	75	90
Dilution neutralisation	5	50	80

Based on the results of the collaborative study, the standard deviation for each method and each product was calculated (table E.3).

Table E.3 - Standard deviation (δ) of \log_{10} reduction - by Method and Product

	Test method	
Product	Membrane filtration	Dilution neutralisation
Phenol	0,50	0,78
Benzalkonium chloride	1,15	1,34

The maximum error on the test result (d) can be estimated using the following equation:

$$d = \sqrt{\frac{t^2_{(1-\rho/2)} \cdot \sigma^2}{n}} \quad (\text{E.1})$$

where

d is the maximum error of the test result;

t is the Student's t value (t is equal to 1,96 when n is large);

n is the number of replicate tests performed;

σ is the standard deviation of the log reduction;

ρ is the type 1 error.

It was decided that the test should provide a 95 % confidence level, which sets σ at 5 %.

n can be deduced as a function of σ and d using following equation:

$$n = \frac{t^2_{(1-\rho/2)} \delta^2}{d^2} \quad (\text{E.2})$$

An extrapolation based on this assumption based on the above equation (E.2), where n is now expressed as a function of σ and d is given in Table E.4.

APPLICATION OF TABLE E.4

According to Table E.4 the conclusions which can be drawn from the results of replicate tests are as follows.

It is possible to assume, as observed in the interlaboratory study, that the standard deviation (δ) is not greater than 1,3, if the average log reduction is the required log reduction (5 in this case) and if the mean is the observed log reduction (6 if the observed reduction is 10^6).

In order to guarantee that the average log reduction is equal to 5 with a confidence level of 95 % (which implies a mean of 6), approximately 6 replicates (n) will need to be performed (Table E.4, standard deviation = 1,3, maximum absolute error on the log reduction is $6-5 = 1$).

With the same assumption as in the previous paragraph, it can be said that:

If a consistent pass value of 5 or more log reduction in viable count is obtained at the highest concentration, it can be concluded (at the 95 % confidence level) that the average log reduction is equal to or greater than:

3,9 if 5 replicates have been performed;

where

$$3,9 = 5 - \sqrt{\frac{(1,96)^2 \times (1,3)^2}{5}} \quad (\text{E.3})$$

3,5 if 3 replicates have been performed; and

2,5 if only 1 replicate has been performed;

NOTE All replicate tests should be carried out using separate subcultures taken from the stock culture as defined in

5.4.1.2. The value of 1.3 for the standard deviation is only valid for replicate test results obtained from separate subcultures taken from the stock culture as defined in **5.4.1.2**. It is not valid for replicate tests carried out using sequential second and third subcultures taken from the stock culture.

Table E.4

Standard deviation	Maximum absolute error on log reduction																
	0,125	0,25	0,375	0,5	0,625	0,75	0,875	1	1,125	1,25	1,5	1,75	2	2,25	2,5	2,75	3
0,5	62	16	7	4	3	2	2	1	1	1	1	1	1	1	1	1	1
0,6	89	23	10	6	4	3	2	2	2	1	1	1	1	1	1	1	1
0,7	121	31	14	8	5	4	3	2	2	2	1	1	1	1	1	1	1
0,8	158	40	18	10	7	5	4	3	2	2	2	1	1	1	1	1	1
0,9	200	50	23	13	8	6	5	4	3	2	2	2	1	1	1	1	1
1	246	62	28	16	10	7	6	4	4	3	2	2	1	1	1	1	1
1,1	298	75	34	19	12	9	7	5	4	3	3	2	2	1	1	1	1
1,2	355	89	40	23	15	10	8	6	5	4	3	2	2	2	1	1	1
1,3	416	104	47	26	17	12	9	7	6	5	3	3	2	2	2	1	1
1,4	482	121	54	31	20	14	10	8	6	5	4	3	2	2	2	1	1
1,5	554	139	62	35	23	16	12	9	7	6	4	3	3	2	2	2	1
1,6	630	158	70	40	26	18	13	10	8	7	5	4	3	2	2	2	2

Annex F
(informative)
**Information on the application and interpretation of European Standard on
chemical disinfectants and antiseptics⁸⁾**

F.1 Application and interpretation of test methods

General guidelines for the application and interpretation of test methods in accordance with European Standards for chemical disinfectants and antiseptics are as follows.

- a) All "use recommendations" for chemical disinfectant and antiseptic products should be supported by results of bactericidal, mycobactericidal, tuberculocidal, fungicidal, yeasticidal, sporicidal and virucidal European Standard tests that are appropriate to the intended field and method of application.
- b) To achieve this, chemical disinfectant and antiseptic products should be subjected to a specified programme of testing that will include phase 1, phase 2 step 1 and phase 2 step 2 tests, except for situations as given in points e), f) and g).
- c) "Use recommendations" may be supported by results of phase 3 tests that are appropriate to the intended field and method of application.
- d) The various steps and phases are defined as follows:
 - phase 1 suspension tests for the basic activity of the product;
 - phase 2 step 1 suspension tests under conditions representative of practical use;
 - phase 2 step 2 other laboratory tests e.g. handwash, handrub and surface tests
simulating practical conditions;
 - phase 3 field tests under practical conditions.
- e) It is accepted that for certain applications, the phase 2 step 1 and phase 2 step 2 tests may provide sufficient information for the particular application and that additional phase 1 tests may not be relevant.

For applications where phase 2 step 1 and phase 2 step 2 tests without phase 1 tests are used to support "use recommendations", the justification for omitting phase 1 tests should be given. Such applications will be indicated either in the European Standard itself or in the additional European Standard that specifies guidelines for the application and interpretation of the tests.

- f) It is accepted that for certain applications, the phase 2 step 1 suspension tests may provide sufficient information for the particular application and that additional phase 2 step 2 tests may not be relevant.

⁸⁾ CEN/TC 216 would like to draw the attention of the reader of this standard to the agreements which were reached concerning the relationship between this European Standard and future European Standards. The guidelines given in this Annex should be followed when using the European Standards on chemical disinfectants and antiseptics.

For applications where phase 2 step 1 tests without phase 2 step 2 tests are used to support use recommendations, the justification for omitting phase 2 step 2 tests should be given. Such applications will be indicated either in the European Standard itself or in the additional European Standard that specifies guidelines for the application and interpretation of the tests.

- g) It is accepted that for certain applications, the phase 2 step 2 together with phase 1 tests may provide sufficient information for the particular application and that additional phase 2 step 1 tests may not be relevant.

For applications where phase 2 step 2 tests without phase 2 step 1 tests are used to support product claims, the justification for omitting phase 2 step 1 tests should be given. Such applications will be indicated either in the European Standard itself or in the additional European Standard that specifies guidelines for the application and interpretation of the tests.

- h) All claims for "bioactive substances" should be supported by appropriate phase 1 tests.

F.2 Guide to interpretation of tests for chemical disinfectants and antiseptics

A separate European Standard (or European Standards) that will be used as a guide to the interpretation of tests for chemical disinfectants and antiseptics will be prepared after the standard test methods have been agreed.

This standard will specify in detail the relationship of the various tests to one another and to "use recommendations"

Bibliography

- [1] European Pharmacopeia (EP), Edition 1997 supplement 2000, Water for injections

Informasi pendukung terkait perumus standar

[1] Komite Teknis Perumus SNI

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